Fig. 3. *Coenobita purpureus.* Maxillules (A–E) and maxillae (F–J) of zoeal stages I–V. Scales 0.1 mm.

Third maxilliped (Fig. 5, B): Now composed of exopod with 5 natatory setae and lobe-like endopod.

Pereiopods (Fig. 1, b): Very small buds found behind third maxilliped in lateral view.

Abdomen (Fig. 1, B): Sixth somite still not separated from telson.

Telson (Fig. 6, B): 8+8 processes including additional pair of short plumose setae; fifth to eighth plumose setae with spinules. Median telson notch indistinct.

Third zoea

Antennule (Fig. 2, C): Two-segmented; distal segment with 5 aesthetascs (one of them short) in addition to 2 short plumose setae; proximal segment with 3 long plumose and 4 short smooth setae and bud of inner flagellum small.
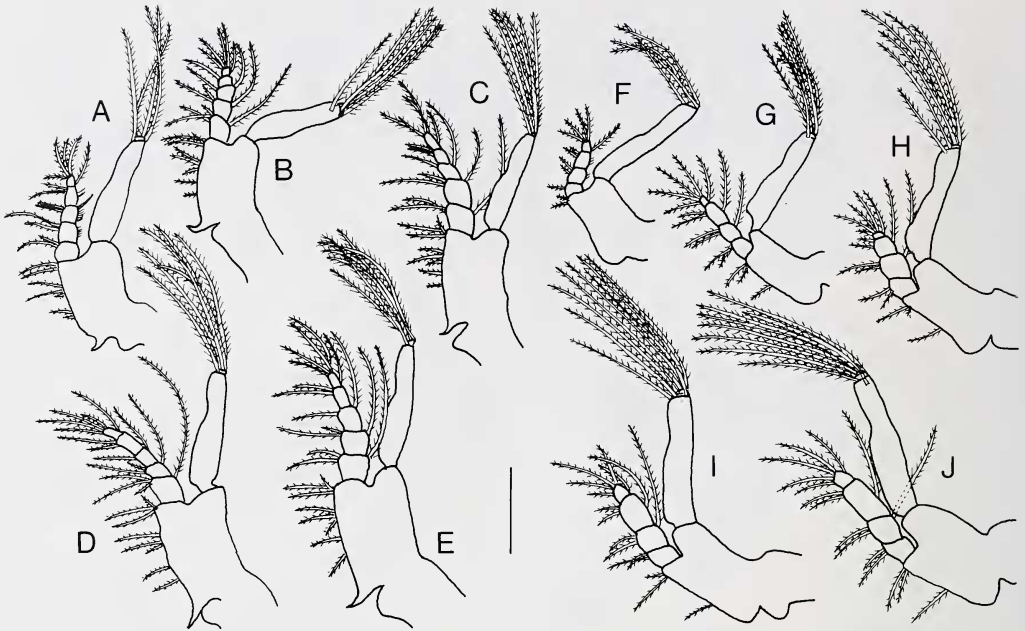
FIG. 4. *Coenobita purpureus*. First (A–E) and second (F–J) maxillipeds of zoeal stages I–V. Scale 0.2 mm.
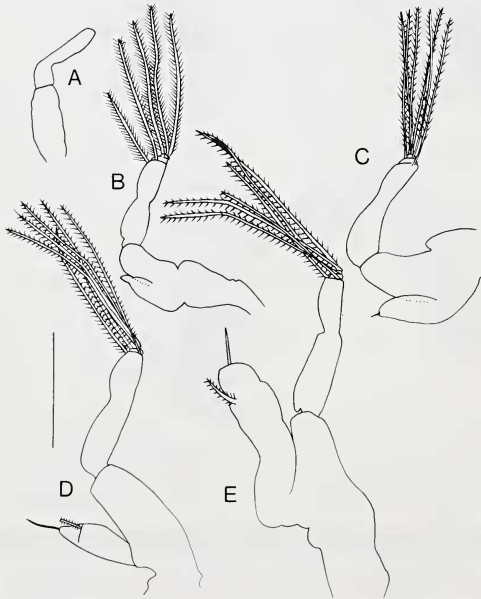

FIG. 5. *Coenobita purpureus*. Third maxillipeds (A–E) of zoeal stages I–V. Scale 0.2 mm.

Antenna (Fig. 2, H): Endopod articulated with basis, bearing a terminal seta. Scale with 13 plumose setae.

Mandible: No observation.

Maxillule (Fig. 3, C): Endopod and basal endite as in preceding stage, but denticulate spines on basal endite relatively large. Coxal endite with 6 plumose and 2 short simple setae.

Maxilla (Fig. 3, H): Setation of endopod as in second zoea. Setal formula of basal endite 5+4, that of coxal endite 9+4. Scaphognathite incomplete, anterior lobe with 10 plumose setae.

First maxilliped (Fig. 4, C): Exopod with 6 natatory setae. Setal formula of five-segmented endopod progressing distally 3–3–2–2–5.

Second maxilliped (Fig. 4, H): As in previous stage.

Third maxilliped (Fig. 5, C): Exopod with 6 natatory setae. Endopod somewhat larger than in previous stage, bearing 1 short terminal seta.

Abdomen (Fig. 1, C): Sixth somite distinct, provided with sharp mediodorsal spine. Sixth somite provided with uropods each consisting of exopod and endopod; exopod with 8 plumose setae, endopod naked, much smaller than exopod.

Telson (Fig. 6, C): 8+1+8 processes; a short median plumose seta flanking 8 processes; first process being a short spine, second a fine simple
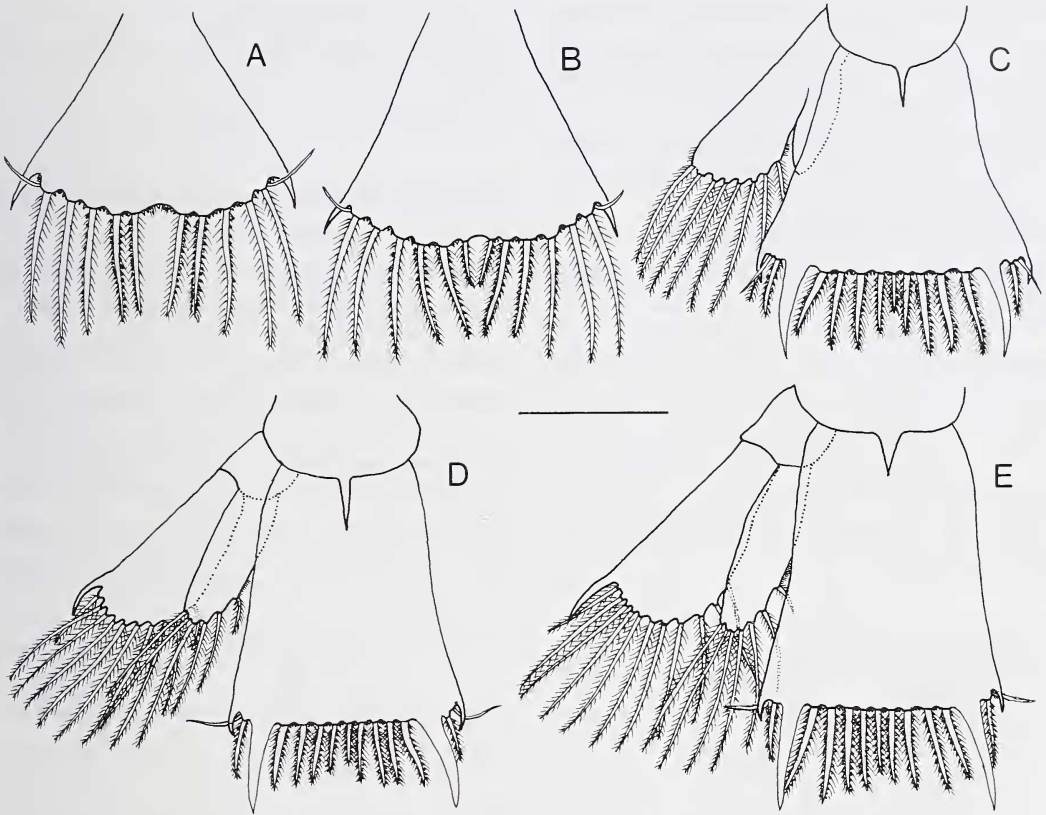
Fig. 6.   *Coenobita purpureus.* Telsons and uropods (A–E) of zoeal stages I–V. Scale 0.3 mm.

hair, third an articulated plumose seta, fourth a large fused spine, fifth to eighth articulated plumose setae; third, fifth to eighth and median plumose setae with spinules.

Fourth zoea

Antennule (Fig. 2, D):   Bud of inner flagellum distinct. Distal segment (outer flagellum) with 4 aesthetascs (two of them long) and 1 long and 1 short plumose setae, proximal segment with 4 very short smooth and 4 long plumose setae (one of them subterminal).

Antenna (Fig. 2, I):   Unchanged.

Mandible: No observation.

Maxillule (Fig. 3, D):   Basal endite with 6 strong denticulate spines and 2 short smooth setae; coxal endite with 6 pronounced and 2 short setae.

Maxilla (Fig. 3, I):   Setation of endopod as in previous stage. Basal and coxal endites with 5+4 and 8+4 setae, respectively.   Anterior lobe of

scaphognathite with 13 plumose setae.

First maxilliped (Fig. 4, D):   Natatory setae as in previous stage. Setal formula of endopod progressing distally 4–3–2–2–5.

Second maxilliped (Fig. 4, I):   Exopod with 8 natatory setae; setation of endopod unchanged.

Third maxilliped (Fig. 5, D):   Nearly as in previous stage, but 1 subterminal plumose seta on endopod.

Abdomen: Exopod and endopod of uropod (Fig. 6, D) distinctly articulated with protopod, exopod provided with 1 sharp posterolateral marginal tooth and 10 plumose setae, endopod with 6 plumose setae.

Telson (Fig. 6, D):   Unchanged.

Fifth zoea

Antennule (Fig. 2, E):   Distal segment (outer flagellum) bearing 4 terminal (one of them short) and 2 subterminal (one of them short) aesthetascs

and 3 plumose setae (one of them long); proximal segment with 4 short smooth and 4 long plumose setae (one of them subterminal). Bud of inner flagellum remained undeveloped.

Antenna (Fig. 2, J): Endopod two-segmented, terminating in 1 seta, scale with 14 plumose setae.

Mandible: No observation.

Maxillule (Fig. 3, E): Unchanged.

Maxilla (Fig. 3, J): Scaphognathite still incomplete, anterior lobe bearing 17 plumose setae.

First maxilliped (Fig. 4, E): Nearly as in previous stage, setal formula of endopod progressing distally 3–3–2–2–5.

Second maxilliped (Fig. 4, J): Unchanged.

Third maxilliped (Fig. 5, E): Similar to previous stage but endopod larger.

Buds of pereiopods and pleopods (Fig. 1, e): More distinct.

Abdomen: Exopod and endopod of uropod (Fig. 6, E) with 11 and 7 plumose setae, respectively.

Telson (Fig. 6, E): Unchanged.

Description of glaucothoe stages of *C. purpureus* and *C. cavipes*

The following description is generalized for two species except differences that are mentioned.

Carapace (Fig. 7, A): Shorter than abdomen including telson. Rostrum rounded, extending beyond front. Eyes and eyestalks well-developed.

Antennule (Fig. 7, B): Bearing unsegmented outer and inner flagella; outer flagellum with 8 aesthetascs and a few setae; inner one with 4 terminal setae in *C. purpureus*, 5 setae (3 terminal and 2 at midlength) in *C. cavipes*; 2 proximal
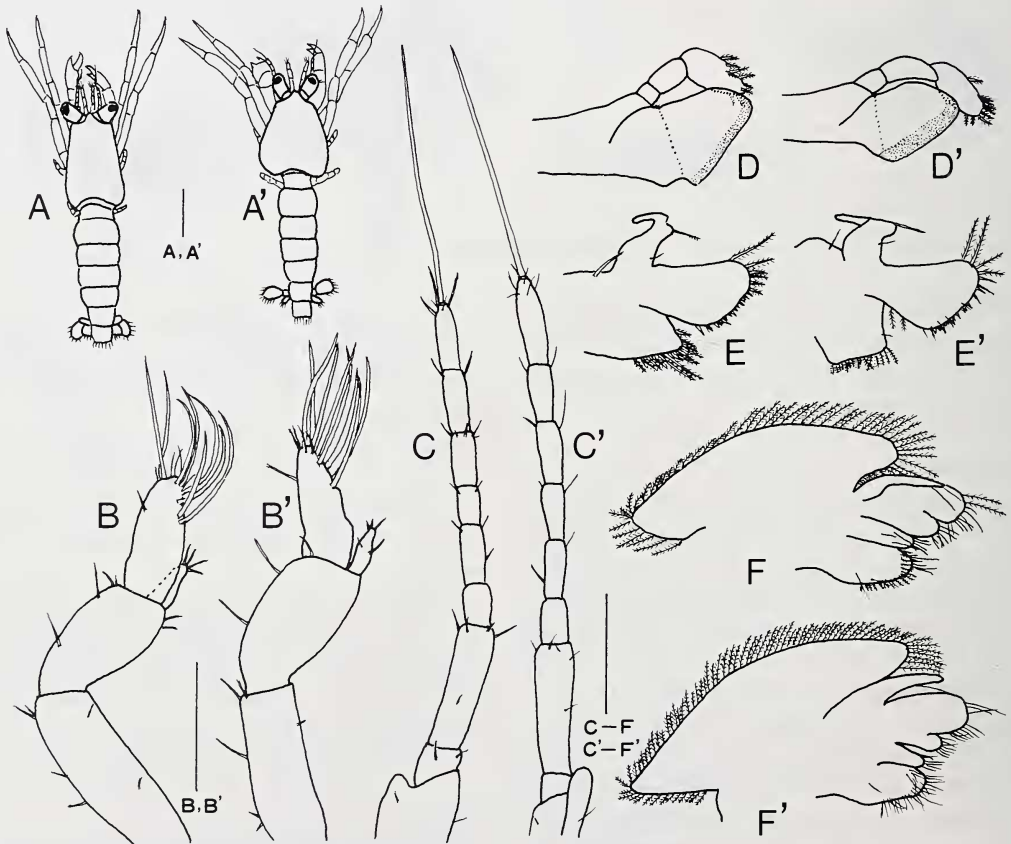

FIG. 7. *Coenobita purpureus* (A–F) and *C. cavipes* (A′–F′), glaucothoes. A, A′, dorsal view; B, B′, antennules; C, C′, antennae; D, D′, mandibles; E, E′, maxillules; F, F′, maxillae. Scales 1.0 mm for A, A′, 0.2 mm for B, B′, C–F, C′–F′.

segments each with a few short setae.

Antenna (Fig. 7, C): Composed of 3 peduncular segments and six-segmented flagellum; terminal flagellar segment with 1 long apical and a few short setae, other segments each with a few setae; scale reduced to a small knob.

Mandible (Fig. 7, D): Palp three-segmented, ultimate segment with 6-9 plumose setae in *C. purpureus*, 8–11 plumose setae in *C. cavipes*; cutting edge of molar process chitinous and brownish.

Maxillule (Fig. 7, E): Endopod unsegmented in *C. purpureus*, two-segmented in *C. cavipes*; bearing 2 short setae, one of them arising from a process on proximal half of unsegmented endopod or proximal segment of two-segmented endopod; basal endite bearing 8 setae (6 plumose and 2 smooth) and 15 spinules in *C. purpureus*, 13 setae (4 plumose and 9 smooth) and 12 spinules in *C. cavipes*; coxal endite with 15 plumose setae in *C. purpureus* and 16 plumose and 1 smooth setae in *C. cavipes*.

Maxilla (Fig. 7, F): Endopod with 2 setae in *C. purpureus*, 1 seta in *C. cavipes*; distal lobe of basal endite with 2 plumose and 8 smooth setae in *C. purpureus*, 10 smooth setae in *C. cavipes*; proximal

lobe with 7 setae; distal lobe of coxal endite with 7 setae in *C. purpureus*, 6 setae in *C. cavipes*; proximal lobe with 16 plumose and 11 smooth setae in *C. purpureus*, 29 smooth setae in *C. cavipes*; scaphognathite with posterior lobe complete, bearing 51 plumose setae in *C. purpureus*, 75 in *C. cavipes*.

First maxilliped (Fig. 8, A): Endopod unsegmented; without setae in *C. purpureus*, bearing 2 terminal setae in *C. cavipes*; exopod bearing 6 lateral marginal plumose setae in *C. purpureus*, 1 short terminal and 7 lateral marginal plumose setae in *C. cavipes*; basal and coxal lobes bearing 18 and 10 plumose setae respectively in *C. purpureus*, 20 and 7 in *C. cavipes*.

Second maxilliped (Fig. 8, B): Endopod four-segmented, each segment bearing a few plumose setae; exopod two-segmented, distal segment bearing 6 plumose setae in *C. purpureus*, 7 in *C. cavipes*.

Third maxilliped (Fig. 8, C): Endopod five-segmented, each segment with a few or numerous setae; exopod three-segmented, ultimate segment setaless in *C. purpureus*, bearing 1 plumose seta in *C. cavipes*.

Chelipeds (Fig. 8, D): Similar and almost

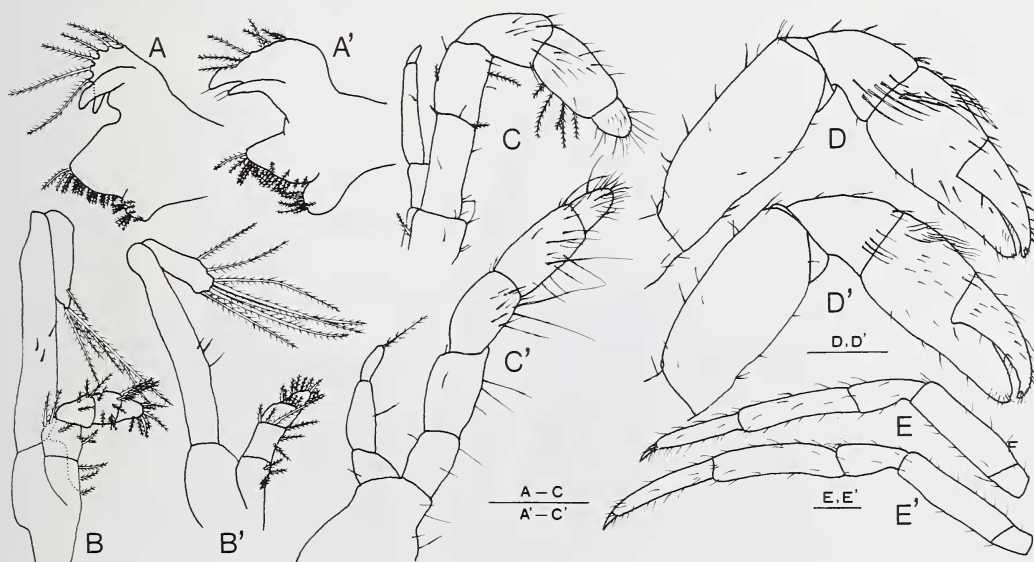FIG. 8. *Coenobita purpureus* (A–E) and *C. cavipes* (A′–E′), glaucothoes. A, A′, first maxillipeds; B, B′, second maxillipeds; C, C′, third maxillipeds; D, D′, left chelipeds; E, E′, second pereiopods. Scales 0.2 mm for A–C, A′–C′, 0.3 mm for D, D′, E, E′.

equal in length; dactylus subequal in length to palm; each segment with scattered setae.

Second (Fig. 8, E) and third pereiopods: Each ending in a corneous claw, bearing a few scattered setae.

Fourth pereiopod (Fig. 9, A): Sparsely setose on each segment; propodus bearing some corneous granules or blunt spines in *C. purpureus*, two rows of spinules in *C. cavipes*; dactylus with 1 long and a

few short setae.

Fifth pereiopod (Fig. 9, B): Distal two segments sparsely setose, some setae long and curved; bearing a few corneous granules and spines in *C. purpureus*, corneous spines only in *C. cavipes*.

Pleopods: Present on second (Fig. 9, C) to fifth abdominal somites; biramous; endopod being a small lobe, bearing 2 short, curved setae subterminally; exopod well-developed, bearing 9 plumose
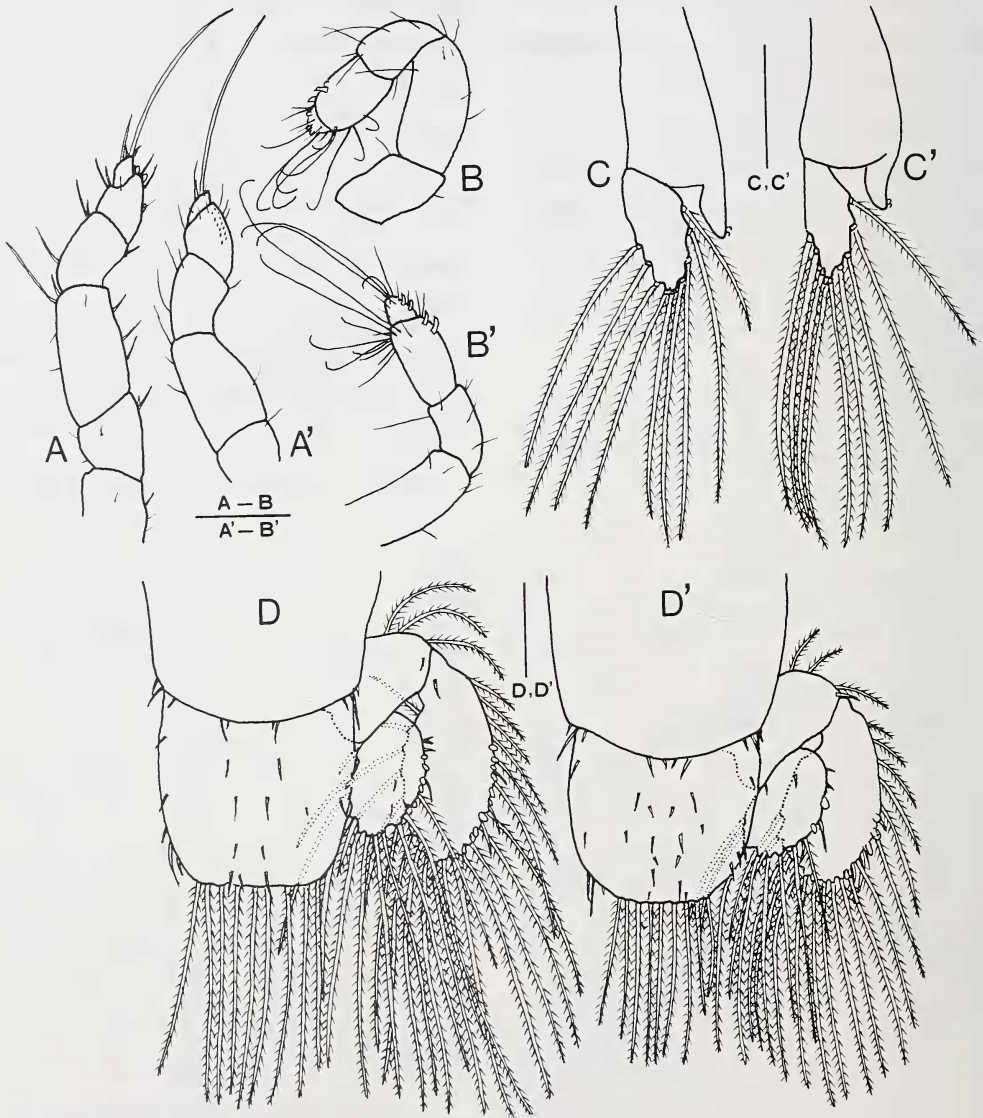

Fig. 9. *Coenobita purpureus* (A–D) and *C. cavipes* (A′–D′), glaucothoes. A, A′, fourth pereiopods; B, B′, fifth pereiopods; C, C′, second pleopods; D, D′, telsons and uropods. Scales. 0.2 mm.

setae.

Abdomen (Fig. 7, A): Dorsal and lateral spines absent. Uropodal exopod (Fig. 9, D) of *C. purpureus* with 22 plumose and a few smooth setae and 11 corneous blunt spines on lateral margin, endopod of same with 11 plumose and a few smooth setae and 8 corneous blunt spines on lateral margin. Setation and spination of uropod in *C. cavipes* somewhat reduced in number, exopod with 18 long plumose and a few short smooth setae and 4 corneous spines, endopod with 8 long plumose and a few short smooth setae and 2 or 3 corneous spines. Protopod of uropod with 3 plumose and 1 short smooth setae on lateral margin; sixth abdominal somite sparsely provided with smooth setae on posterior margin.
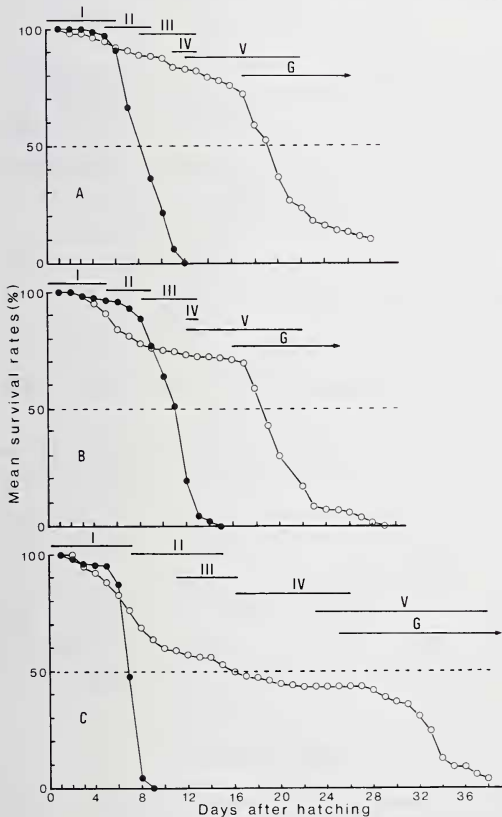

FIG. 10. Survivorship curves for starved (solid circles) and fed (open circles) larvae of *Coenobita purpureus* (A), *C. rugosus* (B) and *C. cavipes* (C) at 28±0.9°C. Horizontal bars (I–V) indicate the duration of zoeal larvae from first to fifth stages; arrows of bars (G) show existence of glaucothoes.

Telson (Fig. 9, D): Bearing 9 long plumose setae on posterior margin, some smooth setae on lateral margin and dorsal surface.

Pereiopods and pleopods well-developed, functional.

Survival rates of larvae of three *Coenobita* species and duration of each zoeal stage

Survivorship curve of larvae of each species and the duration of each zoeal stage in both fed and starved conditions are shown in Figure 10. The starved first zoeal larvae of *C. purpureus*, *C. rugosus* and *C. cavipes* all died in 12, 15 and 9 days after hatching, respectively and none of them molted to the next stage. The mean points of 50% survivorship of starved larvae of *C. purpureus*, *C. rugosus* and *C. cavipes* were about day 8, 11 and 7, respectively. The larvae of *C. rugosus* had greater tolerance for starvation than those of the other two species. The mean points of 50% survivorship of fed larvae were about day 19 in both *C. purpureus* and *C. rugosus* and day 16 in *C. cavipes*. The death rates of the first and second zoeae of *C. cavipes* were higher than those of the other two species, thus their survivorship curve showed a different pattern.

The durations of the first to fifth zoeal stages were 6, 4, 5, 2, 10 days in *C. purpureus*, 5, 4, 5, 1, 10 days in *C. rugosus* and 7, 8, 5, 10, 15 days in *C. cavipes*. The fourth zoeae of *C. rugosus* and *C. purpureus* molted to the fifth zoeae in one or two days. But, most of *C. rugosus* third zoeae directly molted to the fifth zoeae. The duration used here indicates the length of time that all survivors in a certain stage needed till the completion of the succeeding molt, although the speeds of molting differed with individuals.

The appearance of glaucothoes obtained in this study ranged from the 17th to 23rd days of the larval life in *C. purpureus*, from the 16th to 19th days in *C. rugosus* and from the 25th to 38th days in *C. cavipes*. The first glaucothoes emerged on the 5th day after the appearance of the fifth stage zoeae in *C. purpureus*, on the 4th day in *C. rugosus* and on the 2nd day in *C. cavipes*. The survival rates of larvae (including the fifth zoeae and glaucothoes) after the appearance of glaucothoes
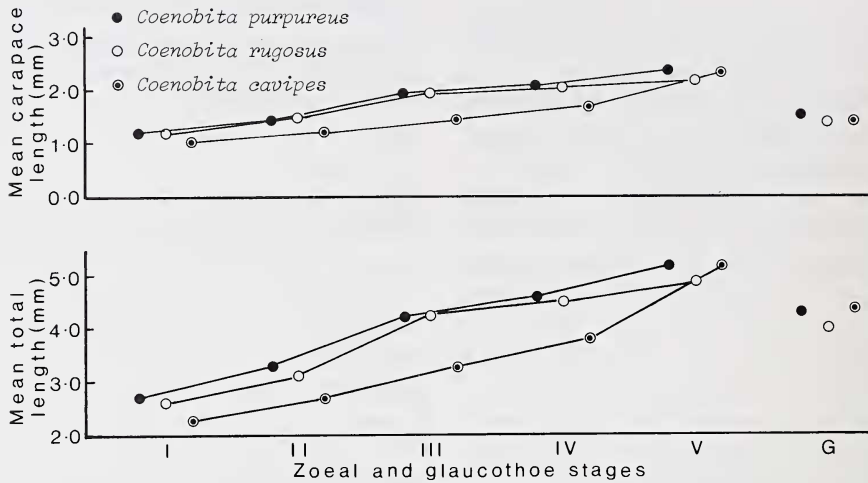
FIG. 11.   Comparison of larval growth rates of three species of *Coenobita*.

rapidly declined in all the three species, as shown in Figure 10. Three of the 8 glaucothoes of *C. purpureus* used in shell utilization experiment entered small shells provided in an aquarium on the 10th day after their appearance, one glaucothoe did not enter shell. For the remaining four it was not certain whether they had died or burried in sand provided in the aquarium.

Total and carapace lengths of each stage zoea and growth factors

The mean total and the mean carapace lengths of each stage zoea of the three *Coenobita* species are summarized in Table 1 and are plotted in Figure 11. Both the total and the carapace lengths of *C. cavipes* zoeae from the first to fourth stages were usually small as compared with those of *C. purpureus* and *C. rugosus*. *C. cavipes* showed nearly a linear growth in both the total and the carapace lengths from the first to fourth zoeal stages. The second stage zoeae of *C. purpureus* and *C. rugosus* rapidly grew when they molted to the third stage, while the fourth stage zoeae of *C. cavipes* did when they molted to the fifth stage. And finally the last (fifth) stage zoeae of *C. cavipes* and *C. purpureus* attained to the same size. Also, the fifth stage zoeae of *C. rugosus* reversely became much smaller than those of *C. purpureus* and *C. cavipes* which are large-sized species. However,

the glaucothoes of *C. rugosus* showed almost the same size as those of *C. cavipes* in carapace length. All the glaucothoes obtained of the three species became smaller than the fifth stage zoeae in both total and carapace lengths, but this was due to inclusion of rostrums for measurement.

The zoeal larvae of *C. purpureus*, *C. rugosus* and *C. cavipes* showed the mean total and the mean growth factors (cf. Gore [8]) of 1.91, 1.18, 1.87, 1.18 and 2.27, 1.23, respectively. These values were calculated using total length. Thus, the greatest total growth factor was 2.27 in *C. cavipes*. Instar growth factors using total length ranged from 1.09 to 1.28 in *C. purpureus*, from 1.06 to 1.36 in *C. rugosus*, and from 1.16 to 1.36 in *C. cavipes*. Both *C. rugosus* and *C. cavipes* had the same greatest instar growth factor (1.36), but the stages were different: between the second and third stages in *C. rugosus*, between the fourth and fifth stages in *C. cavipes*, as shown in Figure 11.

DISCUSSION

As shown in Table 2, zoeal characters in each stage were so similar among the three species that it was difficult to find the recognition characters for each species. Also, there were some observational differences in minor features such as setation of appendages in the same species between Shokita and Yamashiro's [5] and the present studies. The

TABLE 2. Major differences in zoeal characters among *Coenobita rugosus* [5], *C. cavipes* [5] and *C. purpureus*

Item	C. rugosus	C.cavipes	C.purpureus
Zoea I			
Antennular aesthetascs	3(4)	3(3)	3
Setation of antenna			
Endopod and scale	3(2) and 10(10)	3(2) and 10(8)	3 and 10
Setation of maxillule			
Endopod and coxal endite	3(2) and 7(5)	2(2) and 7(5)	2 and 7
Setation of maxilla			
Scaphognathite	5(3)	4(4)	4
Endopod	3+2(3+2)	2+2(4+4)	3+2
Basal endite	4+4(3+3)	4+4(3+2)	3+5
Coxal endite	4+7(3+7)	4+7(2+2)	4+7
Setation of 1st maxilliped			
Exopod	4(3)	4(3)	4
Setation of 2nd maxilliped			
Exopod	4(3)	4(3)	4
Zoea II			
Setation of antenna			
Scale	10(10)	10(10)	10
Setation of maxillule			
Endopod and coxal endite	2(2) and 7(5)	2(2) and 7(4)	2 and 7
Setation of maxilla			
Scaphognathite	6(5)	7(7)	8
Basal endite	4+5(4+3)	4+5(4+3)	3+4
Coxal endite	4+7(3+3)	4+7(2+3)	4+7
Setation of 1st maxilliped			
Exopod	6(3)	6(5)	6
Setation of 2nd maxilliped			
Exopod	6(4)	6(4)	6
Setation of 3rd maxilliped			
Exopod	5(2)	5(4)	5
Zoea III			
Setation of antenna			
Scale	13(13)	12(13)	13
Setation of maxillule			
Endopod and coxal endite	2(2) and 8(6)	2(2) and 7(7)	2 and 8
Setation of maxilla			
Scaphognathite	8(10)	10(7)	10
Basal endite	4+4(4+4)	4+5(4+4)	4+5
Coxal endite	4+7(4+3)	4+7(3+6)	4+9
Setation of 1st maxilliped			
Exopod	6(5)	6(5)	6
Setation of 2nd maxilliped			
Exopod	6(5)	6(5)	6

Table 2. (Continue)

Item	C. rugosus	C.cavipes	C.purpureus
Zoea IV			
Setation of antenna			
Scale	14(14)	15(15)	13
Maxillule			
Spines of basal endite	5(4)	6(5)	6
Setation of maxilla			
Scaphognathite	11(10)	11(12)	13
Setation of 1st maxilliped			
Exopod	6(5)	6(4)	6
Setation of 2nd maxilliped			
Exopod	8(5)	7(6)	8
Zoea V			
Antennular aesthetascs	4+1(5+2)	4+2(5+2)	4+2
Setation of antenna			
Scale	15(15)	16(15)	14
Setation of maxilla			
Scaphognathite	15(11)	17(10)	17
Setation of 1st maxilliped			
Exopod	6(5)	6(6)	6

This table was made on the basis of Shokita and Yamashiro [5].

Figures in parentheses are based on Shokita and Yamashiro's study [5].

first zoea of *C. rugosus* reported by Yamaguchi [3] shares some characters with that of *C. purpureus*. As pointed out by Shokita and Yamashiro [5], Yamaguchi's description on the coxae of the fifth legs in the adult male of *C. rugosus* fits well the character of *C. purpureus*, which differs from that of *C. perlatus* [9]. In Kikaijima Island where Yamaguchi studied *C. purpureus* was the most abundant species, but the other species were not found [10]. Judging from these facts, it seems very likely that the first zoea of *C. rugosus* described by Yamaguchi belongs to *C. purpureus*. The zoeal characters of the three species were nearly similar to those of *C. clypeatus* [4] except minor differences such as the setation of appendages.

The general features of the glaucothoe of the three species are similar, but segmentations and setations of appendages are different, as shown in Table 3. It seems possible that these glaucothoe larvae are separated by the combination of some of the characters listed in it. Yamaguchi's glaucothoe differed from those of *C. purpureus* and *C. rugo-*

sus, both obtained in this study, in that the antenna has seven flagellar segments as in *C. clypeatus* [4] and the antennule has two outer flagellar segments.

Figure 10 shows that the larvae of *C. rugosus* had greater tolerance for starvation than those of the other two species. This may suggest that the larvae of *C. rugosus* have a more fortunate chance of survival when they suffered a temporary food shortage in natural environments. The survival rates of fed zoeal larvae were higher in *C. purpureus* and lowest in *C. cavipes*. Fed fourth zoeae of *C. rugosus* and *C. purpureus* molted to the fifth zoeae in one or two days, but those of *C. cavipes* took seven to ten days. However, the duration of the fifth zoeae was reversely longer in *C. purpureus* and *C. rugosus* than in *C. cavipes*.

According to my interpretation of Provenzano [4], the mean instar growth factor was 1.17 between the first and second stages and 1.11 between the fourth and fifth stages in *C. clypeatus*, in which the greatest instar growth factor was 1.21 between

TABLE 3. Major differences in glaucothoe characters among *Coenobita rugosus*, *C. cavipes* [5] and *C. purpureus*

Item	C. rugosus	C.cavipes	C.purpureus
Antennular aesthetascs	7(6)	8	8
Antenna			
No. of flagellar segments	6(6)	6	6
Mandible			
No. of palpal segments and	2(0) and 5–8(8)	3 and 8–11	3 and 6–9
setae on distal segment			
Maxillule			
Segmentation of endopod	segmented(non)	segmented	non
Setation of maxilla			
Endopod	1(1)	1	2
Scaphognathite	51(50)	75	51
Basal endite	10+5(7+4)	10+7	10+7
Coxal endite	5+21(7+19)	6+29	7+27
Setation of 1st maailliped			
Endopod	0(2)	2	0
Exopod	5+2(9)	7+1	6+0
Basis			
Distal lobe	13(12)	20	18
Proximal lobe	9(11)	7	10
2nd maxilliped			
Segmentation of exopod	3(1)	2	2
Setae on distal segment	7(4)	7	6
of exopod			
3rd maxilliped			
Segmentation of exopod	2(2)	3*	3
Setation of telson	9(11)	9	9
Setation of uropod			
Endopod	10(13)	8	11
Exopod	17(22)	18	22

*) Bearing one terminal plumose seta on distal segment.
Figures in parentheses are based on Shokita and Yamashiro's study [5].

the third and fourth stages; the mean growth factor was 1.17 and the mean total growth factor was 1.85. These values were calculated using total length. In *Birgus latro*, the maximum of instar growth factor was 1.21 between the first and second stages, the mean growth factor was 1.13 and the total growth factor was 1.64, by my interpretation of Reese and Kinzie [6]. The growth factors of five species of Coenobitidae including the above two and the three species of *Coenobita* here studied were 1.18 in mean in-cremental growth and 1.91 in mean total growth, which were nearly similar to the values obtained by Gore [8]. Also, the instar growth factor ranged from 1.06 in *C. rugosus* to 1.36 in both *C. rugosus* and *C. cavipes*. The total growth factor ranged from 1.64 in *B. latro* to 2.27 in *C. cavipes*.

After the appearance of glaucothoes, the survival rates of larvae (including fifth zoeae and glaucothoes) rapidly declined in the three species studied (Fig. 10). This decline was due to predation of fifth zoeae by glaucothoes and cannibalism

of glaucothoes. The death occurred during the molt to glaucothoes also was one of the factor that lowered the survival rates. In *C. purpureus*, the predation of the fifth zoeae by the glaucothoes was the chief (first) factor in the decline of the survival rate and the cannibalism of the glaucothoes was the next factor. The death during the molt was the main factor and cannibalism was the next in *C. rugosus*. Both the death during the molt and cannibalism were the chief factors in *C. cavipes*. It seems likely that both the predation and cannibalism result from the narrow rearing bowl and do not occur in the sea.

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Biochemical Differentiation in Japanese Newts, Genus *Cynops* (Salamandridae)

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ABSTRACT—Genetic variation was surveyed in 38 populations of Japanese newts of the genus *Cynops* using starch gel electrophoresis. *C. ensicauda* from the Ryukyu Archipelago was shown to be genetically well differentiated from *C. pyrrhogaster* from the Japanese main islands. Separation of these two forms at the species level is supported. *C. ensicauda* is genetically divided into two groups, each of which corresponds to previously recognized subspecies. On the contrary, protein variation patterns in *C. pyrrhogaster* are not consistent with the previously recognized subspecies or local races. From available geological information, the electrophoretic clock is calibrated at 1D = 13–22 MY in Japanese *Cynops*.

INTRODUCTION

Two allopatric newt species of the genus *Cynops* are known from Japan. *C. pyrrhogaster* occurs on the main islands of Honshu, Shikoku and Kyushu, while *C. ensicauda* inhabits the Amami and Okinawa Groups of the Ryukyu Archipelago. A marked geographic variation in external morphology has been detected within each species [1, 2]. Some authors [3, 4] considered morphological variations of these two species to overlap with each other and doubted the specific validity of *C. ensicauda*, treating it as a subspecies of *C. pyrrhogaster*. However, only a few comparative studies have been made between these two species [3, 5], and clarification of the taxonomic relationships of these newts requires an extensive survey of geographic variation in Japanese *Cynops* from many approaches.

Although North American and European newt species belonging to *Taricha*, *Notophthalmus*, and *Triturus* have been studied electrophoretically for the purposes of population genetics, taxonomy, and evolutionary biology [6–9], no comparable studies have been done on Asian newts. Available data indicate that genetic distance values calculated between populations can differentiate named species or subspecies and, therefore, seem to provide rough estimates of the limits of species within the family Salamandridae. Thus, an electrophoretic analysis should be a useful tool for investigating taxonomic problems among Japanese newts. The present study was undertaken mainly in order to understand the amount of genetic differentiation between *C. pyrrhogaster* and *C. ensicauda*, as estimated from an electrophoretic analysis of protein variation. Moreover, based upon geological data, we have derived a calibration for the electrophoretic evolutionary clock in Japanese *Cynops* and compare the value with those proposed previously [10, 11].

MATERIALS AND METHODS

A total of 610 newts from 23 populations of *Cynops pyrrhogaster* in western Honshu and Kyushu Islands and 15 populations of *C. ensicauda* in three islands in the Amami Group and four in the Okinawa Group, the Ryukyu Archipelago, were analyzed electrophoretically (Fig. 1 and Table 1). We used southwestern populations of *C. pyrrhogaster* for comparison with *C. ensicauda* since they are geographically adjacent to the range covered by *C. ensicauda*.
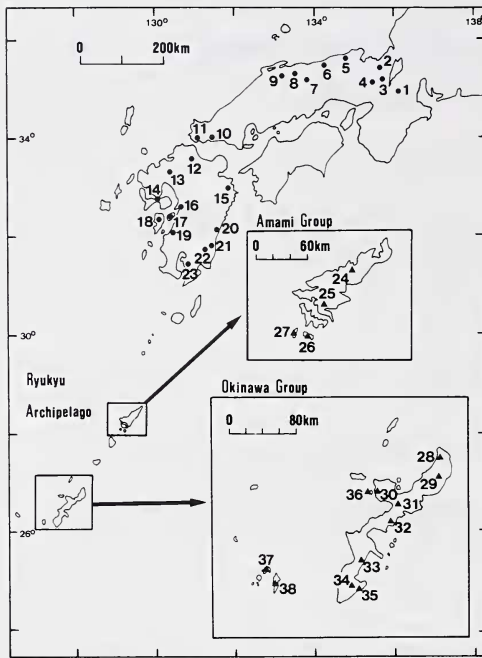
Fig. 1. Geographic localities from which samples of *Cynops* were collected. Localities of *C. pyrrhogaster* are indicated by circles (1–23) and localities of *C. ensicauda* by triangles (24–38).

Samples of liver were removed and maintained frozen at $-84°C$ until used in electrophoresis. Voucher specimens were fixed in 10% formalin, later preserved in 70% ethanol and deposited in Hayashi's collection at Kyoto University. Homogenized tissue extracts were analyzed by standard horizontal starch gel electrophoresis [12–15], using Connaught starch at a concentration of 11.5%. The enzymes examined and locus designations are listed in Table 2. The buffer system employed in the electrophoretic analysis was 0.155 M tris / 0.043 M citrate, pH 7.0 (1:15 dilution of electrode buffer for gel) for all enzymes. Genetic interpretations of allozymic data were based on criteria developed by Selander *et al.* [16]. Enzyme nomenclature and E. C. numbers follow the most recent recommendations of the Nomenclature Committee of the International Union of Biochemistry [17] and abbreviations and isozyme designations follow recommendations of Murphy and Crabtree [18]. Electromorphs were designated by letters with "a" representing the most rapidly

migrating anodal variant.

The unbiased minimum genetic distance between populations (D) recommended by Nei [19] was computed from observed electromorph frequencies. According to Nei's suggestion [19], all negative values obtained using the collection for small sample sizes were regarded as being equal to 0. A UPGMA phenogram [20] was constructed from genetic distances. A contingency Chi-square test was performed to test for inter-sample electromorph frequency heterogeneity [21]. All samples were also tested for conformance to Hardy-Weinberg expectations with the Chi-square test. For statistical tests, $P < 0.05$ was regarded as significant.

RESULTS

A locus was considered polymorphic when two or more electromorphs were detected. Only one of the 15 loci resolved (*Ap-A*) was monomorphic for the same electromorph in all individuals. Table 3 summarizes electromorph frequencies for the remaining 14 polymorphic loci. Fixed differences between *C. pyrrhogaster* and *C. ensicauda* were identified at three loci (*Acp-A*, *Iddh-A* and *M-Me-A*).

Ten of the remaining 11 loci showed significant heterogeneity in electromorph frequencies (Table 4). At four of these 11 loci, a single electromorph predominated in all populations (*Ldh-A*, *Ldh-B*, *M-Mdh-A* and *Pgdh-A*). At *Ldh-A* and *M-Mdh-A* loci, electromorphs other than the common one were unique to single populations. Among the remainder of these four loci, some electromorphs with low to moderate frequency of occurrence were shared among two or more populations. Seven other loci had different variants predominating in different populations (*S-Aat-A*, *Est-1*, *Gpi-A*, *S-Mdh-A*, *S-Me-A*, *Pgm-A*, *S-Sod-A*).

Within *C. pyrrhogaster*, three loci were monomorphic (*Acp-A*, *Ap-A* and *Iddh-A*) and all of 12 polymorphic loci showed statistically significant heterogeneity in electromorph frequencies (Table 4). *C. ensicauda* had six monomorphic loci (*Ap-A*, *Iddh-A*, *Ldh-A*, *M-Mdh-A*, *M-Me-A* and *S-Sod-A*) and significant heterogeneity in electromorph frequencies was observed at seven of nine poly-

TABLE 1. Species, sample size, and locality data for the animals used for electrophoretic analysis

Species	Population number	Locality	N
Cynops pyrrhogaster	1	Shigaraki, Shiga	20
	2	Miyama, Kyoto	39
	3	Kyoto, Kyoto	16
	4	Kameoka, Kyoto	8
	5	Kumihama, Kyoto	4
	6	Tottori, Tottori	19
	7	Ningyo Pass, Okayama	20
	8	Mt. Daisen, Tottori	8
	9	Hirose, Shimane	20
	10	Yamaguchi, Yamaguchi	20
	11	Sanyo, Yamaguchi	20
	12	Yukuhashi, Fukuoka	20
	13	Higashiseburi, Saga	20
	14	Isahaya, Nagasaki	21
	15	Usuki, Oita	5
	16	Shiranui, Kumamoto	20
	17	Kamijima Isl., Amakusa Isls.	18
	18	Shimojima Isl., Amakusa Isls.	20
	19	Minamata, Kumamoto	20
	20	Tsuno, Miyazaki	20
	21	Miyazaki, Miyazaki	17
	22	Tano, Miyazaki	11
	23	Kanoya, Kagoshima	18
Cynops ensicauda	24	Naze, Amami-Oshima Isl.	27
	25	Mt. Kochi, Amami-Oshima Isl.	20
	26	Ukejima Isl.	9
	27	Yorojima Isl.	21
	28	Kayauchibanta, Okinawajima Isl.	5
	29	Mt. Yonaha, Okinawajima Isl.	8
	30	Motobu, Okinawajima Isl.	5
	31	Mt. Nago, Okinawajima Isl.	10
	32	Ginoza, Okinawajima Isl.	10
	33	Nakagusuku, Okinawajima Isl.	18
	34	Tamagusuku, Okinawajima Isl.	10
	35	Chinen, Okinawajima Isl.	9
	36	Sezokojima Isl.	4
	37	Zamamijima Isl.	30
	38	Tokashikijima Isl.	20

Table 2.   Enzymes and loci analysed in Japanese *Cynops*

Enzyme	Enzyme commis- sion number	Locus
Acid phosphatase	3.1.3.2	*Acp-A*
Aminopeptidase	3.4.11.1	*Ap-A*
Aspartate aminotransferase	2.6.1.1	*S-Aat-A*
Esterase	--	*Est-1*
Glucose phosphate isomerase	5.3.1.9	*Gpi-A*
L-iditol dehydrogenase	1.1.1.14	*Iddh-A*
Lactate dehydrogenase	1.1.1.27	*Ldh-A*
Lactate dehydrogenase	1.1.1.27	*Ldh-B*
Malate dehydrogenase	1.1.1.37	*M-Mdh-A*
Malate dehydrogenase	1.1.1.37	*S-Mdh-A*
"Malic Enzyme"*	1.1.1.40	*M-Me-A*
"Malic Enzyme"*	1.1.1.40	*S-Me-A*
Phosphoglucomutase	5.4.2.2	*Pgm-A*
Phosphogluconate dehydrogenase	1.1.1.44	*Pgdh-A*
Superoxide dismutase	1.15.1.1	*S-Sod-A*

Mitochondrial and supernatant loci are denoted by M- and S- prefixes, respectively.
*NADP-dependent malate dehydrogenase

morphic loci. Among Amami Group populations of *C. ensicauda*, another monomorphic locus was recognized (*Est-1*) and five of eight polymorphic loci were significantly heterogeneous. Among Okinawa Group populations, eight loci were monomorphic and four of seven polymorphic loci showed significant heterogeneity.

The proportions of polymorphic loci ranged from 20.0% (populations 1 and 22) to 53.3% (populations 6, 13, 18 and 19) ($\bar{x}=37.7\%$) in *C. pyrrhogaster* and ranged from 13.3% (population 32) to 40.0% (population 33) ($\bar{x}=27.2\%$) in *C. ensicauda* (Table 3). The mean number of electromorphs per locus was 1.44 (range 1.20–1.60) in *C. pyrrhogaster*, and 1.32 (range 1.13–1.47) in *C. ensicauda*. The frequencies of genotypes were in good agreement with Hardy-Weinberg proportion in most cases, but the significant heterozygote deficiencies occurred at the *S-Aat-A* locus in one population (population 33), at the *M-Me-A* locus in five populations (populations 12, 13, 16, 19 and 20) and at the *S-Me-A* locus in six populations (populations 2, 7, 8, 9, 14 and 16).

Figure 2 presents a UPGMA phenogram based on the Nei's D values, which are shown in Table 5.

The first major dichotomy separates populations of *C. ensicauda* from those of *C. pyrrhogaster* with the mean D value between them being 0.356 (range 0.239–0.724). The mean intraspecific D values are 0.035 (range 0–0.133) in *C. ensicauda* and 0.060 (range 0–0.336) in *C. pyrrhogaster*.

The cluster of *C. ensicauda* is divided into two distinct regional groups, with the mean D value between these two groups being 0.078 (range 0.041–0.133). One subcluster is composed of populations from the Amami Group and another of populations from the Okinawa Group. The mean D values are 0.006 (range 0–0.013) within the former and 0.004 (range 0–0.015) within the latter.

The cluster of *C. pyrrhogaster* is also divided into two distinct groups. One subcluster contains three populations from southernmost part of Kyushu (populations 21–23) and another contains all the remaining populations. The mean D value is 0.156 (range 0.044–0.336) between these two groups.

The mean D value between populations of *C. ensicauda* and three southernmost populations of *C. pyrrhogaster* is 0.532 (range 0.326–0.724), while